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SOLUBLE FORMS OF CD40 INHIBIT BIOLOGIC RESPONSES OF HUMAN B CELLS[1]

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We have expressed the CD40 surface Ag as both a soluble 28-kDa molecule and a 57-kDa Fc fusion protein containing the human IgG1 Fc region. Soluble CD40 and the Fc fusion protein inhibited the proliferative response of anti-IgM-activated human B cells to the CD40 mAb G28–5. Similarly, G28–5- and IL-4-induced IgE secretion from PBMC depleted of T cells was effectively blocked by both forms of soluble CD40. Although the soluble constructs of CD40 had only a minimal inhibitory effect on IL-4-mediated proliferation of anti-IgM-activated B cells, IL-4-induced soluble CD23 shedding from both PBMC and T cells depleted of PBMC, and IgE secretion from PBMC, were significantly reduced in a concentration-dependent manner when soluble CD40 was present in the culture. The data presented demonstrate that both soluble forms of the CD40 molecule are biologically active, and suggest that the ligand for CD40 is inducible in IL-4-stimulated cultures and that it mediates both shedding of sCD23 and IgE secretion.

mAb directed against the CD40 surface Ag have been shown to mediate varied functional effects on human B cells. These include induction of homotypic and heterotypic adhesions (1, 2) and increased cell size (2, 3); proliferation of B cells activated with anti-IgM, CD20 mAb, or phorbol ester alone (4–6) or in concert with IL-4 (3, 7); and the production of IgE (8–10), IgG, and IgM (10) from IL-4-stimulated T cell-depleted cultures. In addition, CD40 mAb have been reported to enhance IL-4-mediated soluble CD23/FcεRII release from B cells (11, 12) and to promote B cell production of IL-6 (13). Recently, in the presence of CD_w32^+ adherent cells, human B cell lines have been generated from primary B cell populations with IL-4 and CD40 mAb (14). Furthermore, germinal center centrocytes can be prevented from undergoing apoptosis if they are activated through CD40 and/or receptors for Ag (15).

Optimal stimulation of B cells occurs when the CD40 surface molecule is cross-linked (6). Fab fragments of CD40 mAb induce only a weak biologic response and can inhibit the proliferative signal delivered by intact antibody. The biologic effects of CD40 mAb can be observed at nanomolar concentrations (3, 4), suggesting that human B cells are extremely sensitive to stimulation through CD40. These findings have led to speculation that the CD40 surface molecule may function as a receptor for a ligand yet to be identified.

The cloning of CD40 (16), nerve growth factor receptor (17, 18), and, more recently, two forms of TNF receptor (19–21) has revealed a homology between these molecules and several others including 4-1BB (22), OX40 (23), and most recently CD27 (24) and Fas Ag (25). The fact that three molecules in this family are receptors for cloned ligands strengthens the argument of a ligand-receptor role for CD40.

We have expressed the CD40 molecule as both a soluble 28-kDa protein and as a 57-kDa gene product of the extracellular domain of CD40 fused to the Fc region of human IgG1. The two forms of sCD40[3] not only effectively inhibit the biologic effects of CD40 antibody, but also inhibit IL-4-induced soluble CD23 production and IgE secretion in the absence of CD40 antibody, strongly suggesting that endogenously produced CD40 ligand is present, and has a role, in these responses.

MATERIALS AND METHODS

Plasmid construction. A cDNA encoding a sCD40 was constructed by PCR using the CD40 clone described previously (16) as a template for amplification. The oligonucleotides used were 5′-CCGTCGACCACCATGGTTCGTCTGCC-3′, which introduces a *SalI* site upstream of the initiator methionine of CD40, and 5′-CCGTCGACGTCTAGAGCCGATCCTGGGG-3′, which inserts a termination codon after amino acid 192 of CD40 followed by sites for *XbaI* and *SalI*. The amplified DNA was digested with *SalI* and cloned into pDC406 (26) to construct pDC406/sCD40.

A second cDNA encoding the extracellular domain of CD40 was constructed by PCR amplification from the same template and using the same upstream oligonucleotide primer. The downstream primer was 5′-ACAAGATCTGGGCTCTACGTATCTCAGCCGATCCTGGGGAC-3′, which inserts the amino acids Tyr, Val, Glu, Pro, and Arg after amino acid 193 of CD40. Glu and Pro are the first two amino acids of the hinge region of human IgG1, and are followed by a *BglII* site (see below) that was used to fuse the extracellular domain of CD40 to the remainder of the Fc. A modified cDNA copy of the human IgG1 Fc region (hinge, CH2, and CH3 domains) was isolated by PCR-mediated amplification using cDNA from cells as a template. The oligonucleotide primers used were 5′-TATTAATCATTCAGTAG GGCCCAGATCTTGTGACAAAACTCAC-3′ and 5′-GCCAGCTTAAC TAGTTCATTTACCCGGAGACAGGGAGA-3′.

The amplified DNA contains an introduced *BglII* site near the beginning of the hinge region, which was used to ligate to the extracellular domain of CD40 to construct a sCD40/human IgG1 Fc

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[3] Abbreviations used in this paper: sCD40, soluble CD40; PCR, polymerase chain reaction; CD40.Fc, CD40/hIgG1 fusion protein.

fusion gene. The fusion gene was also cloned into pDC406 to construct pDC406/sCD40.Fc.

Transfection. Plasmids were transfected into CV-1/EBNA cells as described (26), except that cells were transfected in 175-cm^2 flasks or roller bottles. The cells were fed with DMEM containing 0.5% low IgG FBS (Hyclone, Logan, UT), and supernatants were harvested weekly for up to 4 wk after transfection.

Other reagents. Human rIL-4 was purified from yeast supernatant as described previously (27). The CD40 mAb G28-5 was produced as described previously (4). For B cell proliferation assays, cells were activated by cross-linking surface IgM with rabbit anti-human IgM bound to acrylamide beads (Bio-Rad, Richmond, CA). Human IL-4R.Fc and IL-7R.Fc fusion proteins containing the human IgG1 Fc region were kindly provided by Dr. L. Lauffer (Behringwerke AG, Marburg, Germany). Soluble murine IL-4R was produced as described previously (28).

Preparation of a G28-5 affinity matrix and affinity purification of sCD40 and CD40.Fc. The affinity-purified mouse IgG1 anti-human CD40 mAb (clone G28-5), free of transferrin and other contaminants, was coupled to AminoLink coupling gel (Pierce, Rockford, IL) according to the manufacter's instructions. The final gel preparation contained 1 mg of covalently bound G28-5 antibody per ml of bed resin. The recombinant sCD40 was derived from CV-1/EBNA cells transfected with the plasmid construct containing the human sCD40 gene.

The sCD40-containing supernatants were applied to a 1-ml G28-5-affinity column (1.7 cm × 10.7 cm) at 4°C with an approximate flow rate of 50 ml/h. The column was washed sequentially with 20 ml of 0.5 M NaCl in pyrogen-free PBS and 10 ml of pyrogen-free PBS (Gibco, Grand Island, NY). Bound recombinant sCD40 was eluted with 25 mM citrate, pH 2.8, and collected in 0.5-ml fractions by gravity flow into polypropylene tubes containing 50 μl of 500 mM Hepes, pH 9.1. All wash, elution, and neutralization buffers were 0.2-μm filtered and handled under aseptic conditions before use. Protein-containing fractions were pooled and aliquots removed for G28-5 binding assays and analysis by SDS-PAGE according to the method of Laemmli (29). Proteins were visualized by silver staining. Sample protein concentration was determined using the micro-BCA assay (Pierce) with ultrapure BSA as standard. sCD40 purity and protein concentration were confirmed by amino acid analysis. Purified recombinant sCD40 was absorbed to polyvinylidene difluoride paper and the filter subjected to automated Edman degradation on an Applied Biosystems model 477A protein sequencer essentially as described (30) for amino terminal sequencing.

In the same manner, recombinant human sCD40.Fc was purified from CV-1/EBNA cells transfected with the plasmid construct containing the human sCD40/human IgG1 Fc fusion gene (CD40.Fc). The CD40.Fc-containing supernatants were 0.45-μm filtered and applied to a 0.5-ml protein A/G (Schleicher and Schuell, Keene, NH) antibody-affinity column (1.5 cm × 12.0 cm) at 4°C with a flow rate of 80 ml/h. The column was washed sequentially with 0.5 M NaCl in pyrogen-free PBS until free protein was undetectable in the wash buffer and then with 10 ml of pyrogen-free PBS. Bound recombinant CD40.Fc was eluted and characterized in the same manner as sCD40. For in vitro cell-culture experiments, sCD40 or CD40.Fc were diluted in 10× pyrogen-free PBS or dialyzed overnight at 4°C against 1× pyrogen-free PBS. Endotoxin levels were determined by the Limulus amoebocyte lysate assay (M.A. Bioproducts, Walkersville, MD). All preparations of sCD40 and CD40.Fc contained <1 pg LPS endotoxin/μg protein.

Solid phase binding assay for sCD40 and CD40.Fc. The activity and level of sCD40 and CD40.Fc were measured with the use of a solid-phase binding inhibition assay. Briefly, 96-well enzyme immunoassay plates (Corning, New York, NY) were coated with G28-5 (10 μg/ml in PBS) for 1 h at 37°C, incubated with 1% nonfat dry milk in PBS for 1 h at room temperature, washed once with PBS + 0.05% Tween-20, and then washed five times with PBS. The wells were then incubated with biotinylated sCD40 at a concentration of 5×10^{-10} M in a diluent that consisted of 10% normal goat serum in PBS for 1 h at room temperature. The purified sCD40 or CD40.Fc was biotinylated at a 6:1 biotin to protein molar ratio essentially as described previously (31). A standard curve was then generated by titrating unlabeled sCD40 in duplicate into wells containing biotinylated sCD40 beginning at a concentration of 1×10^{-8} M with serial twofold dilutions. Samples containing unknown levels of sCD40 were also assayed in duplicate wells in the presence of 5.2×10^{-10} M biotinylated sCD40. The plates were then washed extensively with Tween-free PBS. Bound biotinylated sCD40 was detected with streptavidin-horseradish peroxidase (Zymed, San Francisco, CA), washed, and then developed with the use of the TMB Microwell peroxidase substrate system (Kirkegaard and Perry, Gaithersburg, MD). Data reduction was accomplished using the DeltaSoft 2.1 ELISA analysis program for the Macintosh (Biometallics, Inc., Princeton, NJ). Binding data were analyzed using a one-site competitive inhibition equation as described (32).

Cell separation. Human PBMC were isolated from the blood of healthy donors by centrifugation over Histopaque (Sigma Chemical Co., St. Louis, MO). T cell-depleted preparations were obtained by removal of T cells by rosetting with 2-aminoethylisothiouronium bromide-treated SRBC and additional centrifugation over Histopaque. The resulting population was <1% $CD3^+$ as determined by flow cytometry (FACScan; Becton Dickinson, Mountain View, CA). Tonsillar tissue was gently teased and the resulting cell suspension centrifuged over Histopaque. Purification of tonsil B cells was achieved by removal of cells rosetting with 2-aminoethylisothiouronium bromide-treated SRBC and treatment of remaining cells with B cell Lympho-kwik (One Lambda Inc., Los Angeles, CA) for 1 h at 37°C to lyse contaminating non-B cells. The resultant B cell population was >98% $CD20^+$ as determined by flow cytometry.

Culture conditions. All cell cultures were conducted in RPMI + 10% heat-inactivated FCS at 37°C in a humidified atmosphere of 10% CO_2. For the measurement of proliferation, 1×10^5 cells/well were cultured in triplicate in flat bottomed 96-well microtiter plates (Corning) for 72 h in the presence of the appropriate additives as described in *Results*. Cells were pulsed with 1 μCi/well [^{3}H]TdR (25 Ci/mmol; Amersham, Arlington Heights, IL) for the final 8 h of culture. Cells were harvested onto glass fiber disks with an automated cell harvester. Incorporated cpm were measured by liquid scintillation spectrometry.

For sCD23 induction and IgE secretion, 1×10^5 cells/well were cultured in triplicate in round bottomed 96-well Nunc microtiter plates (Intermountain Scientific Corp., Bountiful, UT) for the time indicated in the presence of additives as detailed in *Results*. Experiments to measure IgE secretion in response to G28-5 mAb were conducted using cells from single donors. To examine the effects of sCD40 constructs on IL-4-induced sCD23 and IgE production in the absence of G28-5, pooled cells from selected pairs of donors were used to enhance the overall levels of sCD23 and IgE. Soluble CD23 levels were determined by ELISA using a commercial sCD23 detection kit (The Binding Site, San Diego, CA). The limit of sensitivity of the sCD23 ELISA was 500 pg/ml. IgE was detected by ELISA as follows: flat bottomed 96-well microtiter plates (Corning) were coated with mouse mAb anti-human IgE (Zymed) at a 1:500 dilution in PBS. After washing three times, a blocking step was carried out using 5% nonfat dried milk. This was followed by the addition of a titration of IgE standard (Calbiochem, La Jolla, CA) or test supernatants. After washing three times, biotinylated goat anti-human IgE (Kirkegaard and Perry) was added at 1:500 dilution followed by additional washing and addition of streptavidin-horseradish peroxidase (Zymed), 1:500 dilution. After washing, the reaction was developed using TMB substrate (Kirkegaard and Perry) and OD measured at 520 nm. All incubation steps were performed using a volume of 100 μl/well for 1 h at room temperature. Wash steps were carried out in PBS + 0.05% Tween. The sensitivity of the IgE ELISA was 100 pg/ml.

RESULTS

Expression and purification of sCD40 and CD40.Fc. The sCD40 was purified from CV-1/EBNA cell culture supernatants by G28-5 affinity chromatography. SDS-PAGE of the purified sCD40 in the presence of 1 M dithiothreitol showed a single band of m.w. 28,100 (Fig. 1). In the absence of reducing agent, SDS-PAGE analysis of sCD40 revealed a single band of m.w. 55,200 (data not shown). This banding pattern indicates that the sCD40 exists as a disulfide-linked homo-dimer in solution.

The CD40.Fc was purified from CV-1/EBNA cell culture supernatants by protein A/G affinity chromatography. Gel-electrophoretic analysis of the purified CD40.Fc under reducing conditions showed a single band of m.w. 57,300 (Fig. 2). When SDS-PAGE analysis of CD40.Fc was performed under nonreducing conditions, a single band of m.w. 104,000 was observed (data not shown). As was noted with sCD40, this banding pattern shows that sCD40.Fc exists in solution as a disulfide-linked homodimer.

Solid-phase binding of sCD40 to G28-5. The binding activity of sCD40 was measured by competition of biotin-labeled sCD40 binding to the mAb G28-5 (Fig. 3*A*). In

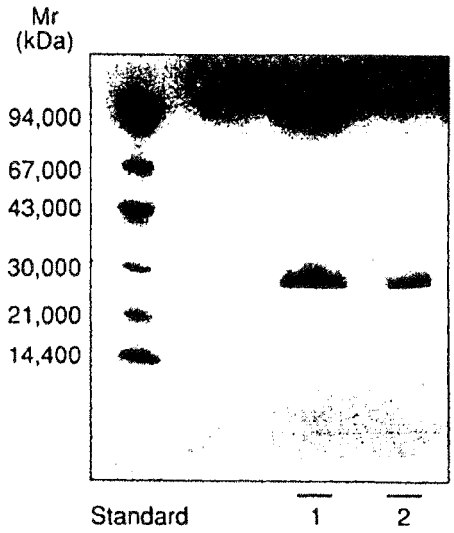

Figure 1. SDS-PAGE of sCD40. Purified sCD40 was denatured under reducing conditions and applied to 8 to 25% gradient SDS-PAGE gel. *Lane 1* was loaded with ~120 ng of sCD40, and *lane 2* was loaded with ~90 ng of sCD40. The gel was electrophoresed and the protein detected by silver staining. The m.w. values for silver-stained bands were determined by comparing relative mobility values for the sample protein with a standard curve generated by a linear least-squares fit of the relative mobility values obtained from the silver m.w. standards.

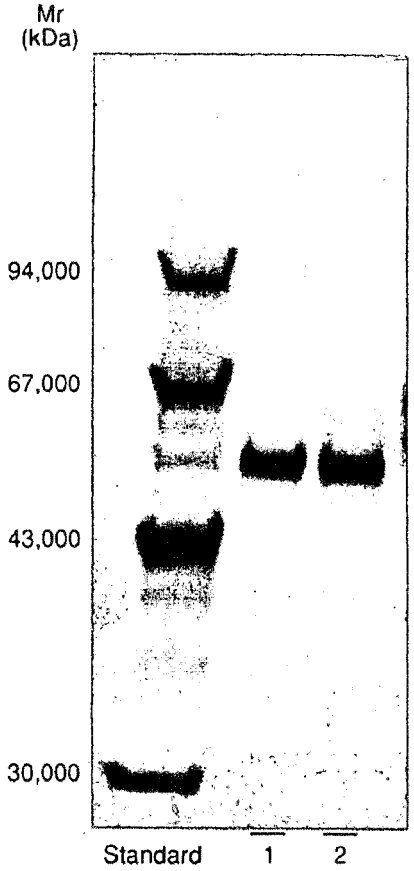

Figure 2. SDS-PAGE of CD40.Fc. Purified CD40.Fc was denatured under reducing conditions and applied to 10% SDS-PAGE gel. *Lane 1* was loaded with 150 ng of CD40.Fc, and *lane 2* was loaded with 150 ng of CD40.Fc from different pooled elution fractions. The gel was electrophoresed and the protein detected by silver staining. The m.w. values for silver-stained bands were determined by comparing relative mobility values for the sample protein with a standard curve generated by a linear least-squares fit of the relative mobility values obtained from the silver m.w. standards.

this experiment (representative of several performed), biotinylated sCD40 (5.2×10^{-10} M) gave an OD maximum of 0.7 ± 0.09 in the presence of $<1.6 \times 10^{-10}$ M unlabeled sCD40. Binding of biotinylated sCD40 was effectively inhibited by unmodified sCD40 in a concentration-dependent manner with complete inhibition occurring in the presence of 1×10^{-8} M unlabeled sCD40. The binding of 5.2×10^{-10} M biotinylated sCD40 to G28-5 was inhibited 50% by unlabeled sCD40 at a concentration of 6.5×10^{-10} M (Fig. 3*B*). This binding inhibition with unlabeled sCD40 is indicative of high affinity binding of sCD40 to G28-5 with an apparent inhibition constant of 3.4×10^{9} M^{-1}. Binding inhibition with unlabeled CD40.Fc revealed that CD40.Fc bound to G28.5 with an affinity that was approximately twofold higher than that for sCD40 (data not shown).

Effect of sCD40 on G28-5-induced B cell stimulation. sCD40 and CD40.Fc were examined for their ability to inhibit G28-5-induced proliferation of tonsil B cells activated with anti-IgM (Fig. 4*A*). Both forms of the sCD40 molecule inhibited proliferation in a concentration-dependent manner, whereas a control Fc fusion protein, human IL-7 receptor.Fc (IL-7R.Fc), and a soluble receptor control, murine IL-4R (data not shown), had minimal effects on [^{3}H]TdR incorporation. The level of inhibition of G28-5-induced B cell proliferation observed with both forms of sCD40 when compared on a molar basis was similar. Neither form of sCD40 had an obvious inhibitory effect on B cell proliferation induced by anti-IgM and IL-4 (Fig. 4*B*), although in three experiments of four performed, a small decrease in the level of [^{3}H]TdR incorporation was consistently observed, compared to that seen with the control IL-7R.Fc. In contrast, IL-4R.Fc inhibited IL-4-induced proliferation in a concentration-dependent manner.

In a second assay system, T cell-depleted PBMC from single donors were cultured in the presence of IL-4 and G28-5, and the culture supernatants tested for the accumulation of secreted IgE after 12 days of culture (Fig. 5). Both sCD40 and CD40.Fc inhibited IgE secretion induced by G28-5, whereas IL-7R.Fc (and mIL-4R; data not shown) had no effect.

Effect of sCD40 in the absence of CD40 mAb. It is a reasonable assumption that at least some of the biologic activities ascribed to CD40 mAb will be shared by the ligand for CD40. It was of interest to determine whether sCD40 or the Fc construct of CD40 could modulate biologic responses of human B cells in the absence of CD40 mAb; in other words, whether certain responses were mediated through the endogenous production of CD40 ligand.

sCD40 and CD40.Fc were tested for their ability to inhibit IL-4-induced IgE secretion from pooled PBMC from pairs of selected donors (Table I). In three experiments, sCD40 inhibited IgE production in a dose-dependent fashion. In an additional experiment, sCD40 was added at a single high concentration (10 μg/ml) and completely inhibited IgE secretion. Addition of CD40.Fc, but not IL-7R.Fc, had a similar inhibitory effect to that observed with sCD40. IgE secretion from IL-4-stimulated single donor PBMC was affected similarly, although the

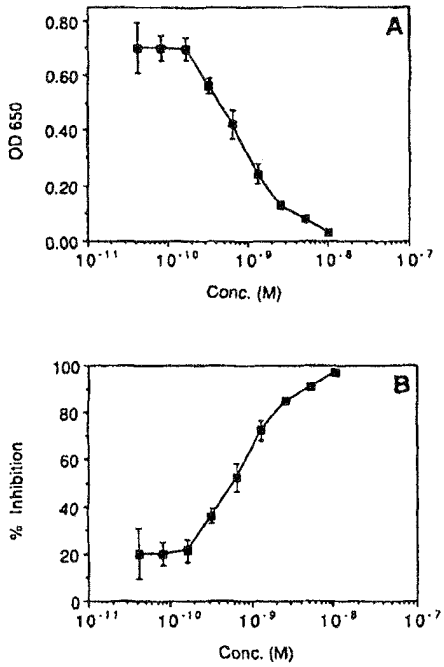

Figure 3. Solid-phase binding of biotinylated sCD40 to G28–5. A constant concentration of biotinylated sCD40 (5.2×10^{-10} M) was incubated at 25°C in the presence of immobilized CD40 mAb G28–5. The unlabeled sCD40 was then titrated into the incubation mixture up to a concentration of 1×10^{-8} M (*A*). The activity of the unlabeled sCD40 was determined by its ability to inhibit binding of biotinylated sCD40 to G28–5 (*B*). In this assay, the maximal OD in the absence of any inhibitor was 0.87.

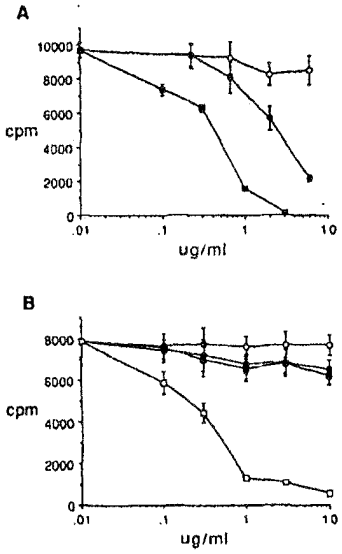

Figure 4. A, sCD40 inhibit G28–5-mediated proliferation. Tonsil B cells (1×10^5/well) were cultured in triplicate for 72 h in the presence of 10 μg/ml anti-IgM and 200 ng/ml G28–5, together with sCD40 (■), CD40.Fc (●), or IL-7R.Fc (○) at the concentrations shown. Results are expressed as mean cpm ± SEM from a representative experiment of three performed. *B*, sCD40 do not inhibit IL-4-induced B cell proliferation. Tonsil B cells (1×10^5/well) were cultured in triplicate for 72 h in the presence of 20 ng/ml anti-IgM and 10 ng/ml IL-4, together with sCD40 (■), CD40.Fc (●), IL-7RFc (○), or IL-4R.Fc (□) at the concentrations shown. Results are expressed as mean ± SEM from a representative experiment of four performed.

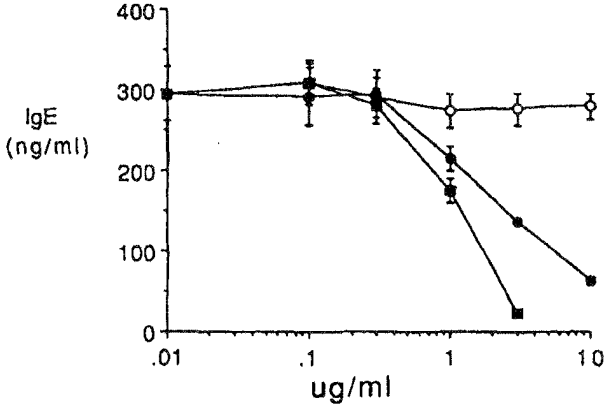

Figure 5. sCD40 inhibit G28–5 mediated IgE secretion. T cell-depleted PBMC (1×10^5/well) were cultured in triplicate in the presence of 5 ng/ml IL-4 and 500 ng/ml G28–5, together with sCD40 (■), CD40.Fc (●), or IL-7R.Fc (○) at the concentrations shown. IgE levels in culture supernatants were measured at day 12. Results are expressed as mean ± 1 SD from a representative experiment of three performed.

overall level of IgE detected was consistently lower (data not shown).

Levels of soluble CD23 were measured in the same culture system at day 6 and day 12 (Table II). The presence of CD40.Fc in IL-4-stimulated cultures resulted in a small, but reproducible, decrease in the sCD23 level at day 6 compared to that seen with cells cultured in IL-4 alone. However, a stronger inhibitory effect was discernable at day 12 of the same cultures. Soluble CD23 induction by IL-4-stimulated T cell-depleted PBMC was affected similarly by the addition of CD40.Fc, causing a small decrease in sCD23 levels at day 6 and a more pronounced inhibition at day 12. In both sCD23 induction systems, sCD40 had a comparable effect to that seen with CD40.Fc at both time points (data not shown).

DISCUSSION

We have expressed the CD40 Ag as both a 28-kDa soluble protein and a 57-kDa Fc fusion protein. Both forms of sCD40 effectively neutralize the biologic activity of the CD40 mAb G28–5 in human B cell proliferation and IgE secretion assays. The relative potency of sCD40 and CD40.Fc was similar when they were compared on a molar basis. While forms of sCD40 have only a minimal inhibitory effect on IL-4-mediated B cell proliferation at day 3, IL-4-induced sCD23 induction and IgE production at day 12 in the absence of CD40 mAb are effectively inhibited, suggesting that these responses are at least partially mediated through the expression of CD40 ligand.

It has been reported that IL-4-induced IgE secretion is dependent on the presence of T cells (33, 34). Inasmuch as IgE production can be induced from T cell-depleted PBMC by addition of CD40 mAb (8, 9), it is possible that the major T cell component required for IgE synthesis is CD40 ligand. Alternatively, since the CD40-mediated signal can promote interactions between B cells and T cells (13), blocking the action of CD40 ligand may prevent key T cell-B cell signaling. Similarly, the inhibitory effects of sCD40 on sCD23 induction from IL-4-stimulated PBMC point to CD40 ligand as an effector molecule in this response. However, sCD23 can also be induced by IL-4 in the absence of T cells, and the inhibition of this by sCD40

TABLE I

sCD40 inhibits IL-4-induced IgE secretion

Addition	IgE (ng/ml)[a]			
	Experiment 1	Experiment 2	Experiment 3	Experiment 4
Medium	<0.1	<0.1	<0.1	<0.1
IL-4	24	47	54	46
IL-4 + sCD40 (0.1 μg/ml)	19	ND	38	ND
IL-4 + sCD40 (0.3 μg/ml)	14	29	24	ND
IL-4 + sCD40 (1 μg/ml)	10	24	8	ND
IL-4 + sCD40 (3 μg/ml)	7	19	2	ND
IL-4 + sCD40 (10 μg/ml)	ND	ND	ND	<0.1
IL-4 + CD40.Fc (10 μg/ml)	ND	ND	ND	18
IL-4 + IL-7R.Fc (10 μg/ml)	21	ND	58	42
IL-4 + control buffer[b]	ND	44	ND	40

[a] IgE levels were measured after 12 days of culture in the presence or absence of 5 ng/ml IL-4 and sCD40, CD40.Fc, or IL-7R.Fc at the concentrations indicated.

[b] Appropriately diluted buffer used to elute and neutralize sCD40 and CD40.Fc (see *Materials and Methods*).

TABLE II

Soluble CD40 inhibits sCD23 shedding

	sCD23 (ng/ml)[a]					
	Exp. 1		Exp. 2		Exp. 3	
	Day 6	Day 12	Day 6	Day 12	Day 6	Day 12
T cell-depleted PBMC + medium	55	<0.5	24	10	10	5
+ IL-4[b]	115	55	96	62	44	27
+ IL-4 + CD40.Fc (1 μg/ml)	ND	ND	88	36	38	9
+ IL-4 + CD40.Fc (3 μg/ml)	97	4	82	31	40	4
+ IL-4 + CD40.Fc (10 μg/ml)	ND	ND	72	28	ND	ND
+ IL-4 + IL-7R.Fc (3 μg/ml)	111	48	103	67	40	22
+ IL-4 + control buffer[c]	ND	ND	93	63	ND	ND
PBMC + medium	12	<0.5	15	5	3	10
+ IL-4	39	25	47	22	48	26
+ IL-4 + CD40.Fc (1 μg/ml)	ND	ND	44	18	46	18
+ IL-4 + CD40.Fc (3 μg/ml)	24	6	37	11	45	12
+ IL-4 + CD40.Fc (10 μg/ml)	ND	ND	28	5	ND	ND
+ IL-4 + IL-7R.Fc (3 μg/ml)	35	26	43	20	50	23
+ IL-4 + control buffer	ND	ND	46	20	ND	ND

[a] Results expressed as mean values of triplicate cultures. The SD of each value in these experiments was always <10% of the mean.

[b] IL-4 was added at a final concentration of 5 ng/ml.

[c] See Table I, footnote *b*.

suggests that IL-4 stimulation also leads to the production of CD40 ligand from non-T cells. The observation that IgE secretion is not detectable in the absence of T cells suggests that, in contrast to signaling with CD40 mAb, stimulation with IL-4 and non-T cell-derived CD40 ligand alone is insufficient for the production of IgE, and an additional component provided by T cells is required.

Although CD40.Fc had a small inhibitory effect on sCD23 levels measured at day 6, it was strongly inhibitory at day 12, suggesting that CD40 ligand was being produced, and having its effect, predominantly in the later stages of the culture period. Both B cells and monocytes show increased surface expression and shedding of CD23 upon activation with IL-4[4] (35, 36). It is possible, therefore, that during the first few days of culture, both cell types are responding directly to IL-4 by producing elevated levels of sCD23, and that in the later period of culture continued expression of sCD23 mainly results from CD40 ligand produced by monocytes or NK cells acting on the B cell population. This is supported by the observation that sCD23 induction by highly purified tonsil B cells stimulated with IL-4 is not inhibited by sCD40 (data not shown), arguing against CD40 ligand being a B cell-derived autocrine factor.

We have found that constructs of sCD40 form homodimers in solution. It has been suggested that dimerization of surface-expressed CD40 can also occur (37), which is a feature shared by other members of the TNF receptor/CD40 family (21). Based on the observation that ligands for this family of receptors can also be oligomeric (38), it is tempting to speculate that multimeric forms of CD40 ligand may exist. The sCD40 molecule will prove to be valuable in determining the range of activities of the CD40 ligand.

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